EXHIBIT 94

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[^{3}H]PtdIns hydrolysis in postmortem human brain membranes is mediated by the G-proteins $G_{q/11}$ and phospholipase C-β

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A method utilizing exogenously added [^{3}H]PtdIns incubated with membranes prepared from postmortem human brain has been shown to provide a means of measuring agonist-induced, guanosine 5′-*O*-(thiotriphosphate) (GTP[S])-dependent hydrolysis of [^{3}H]PtdIns, thus allowing investigations of the activity of the phosphoinositide second-messenger system in accessible human brain tissue. Agonists inducing [^{3}H]PtdIns hydrolysis include carbachol, *trans*-1-aminocyclopentyl-1,3-dicarboxylate (ACPD; a glutamatergic metabotropic receptor agonist), serotonin and ATP, with the latter two agonists producing the largest responses. In addition to ATP, [^{3}H]PtdIns hydrolysis was induced by ADP and by 2-methylthio-ATP, indicating that P_2-purinergic receptors mediate this process. Subtype-selective antibodies were used to identify $G_{q/11}$ and phospholipase C-β as the G-protein and phospholipase C subtypes that mediated GTP[S]-induced and agonist-induced [^{3}H]PtdIns hydrolysis. These results demonstrate that this method reveals that agonist-induced, GTP[S]-dependent [^{3}H]PtdIns hydrolysis is retained in postmortem human brain membranes with properties similar to rat brain. This method should allow studies of the modulation of phosphoinositide hydrolysis in human brain and investigations of potential alterations in postmortem brain from subjects with neurological and psychiatric diseases.

INTRODUCTION

Receptor-coupled, G-protein-dependent activation of phosphoinositide hydrolysis by phospholipase C is a major signal-transduction mechanism in mammalian brain [1]. The phosphoinositides cleaved by phospholipase C include PtdIns, PtdIns4*P* and PtdIns(4,5)P_2, the latter resulting in the formation of two second messengers, Ins(1,4,5)P_3, which releases intracellular sequestered Ca^{2+}, and diacylglycerol, which contributes to the activation of protein kinase C [1]. Although much information has been obtained about the function of this system and its modulation in animal tissues, investigations with human brain have been prevented by tissue deterioration occurring during postmortem delay until freezing [2]. An important goal of research on phosphoinositide metabolism is to develop methods that can be applied to postmortem human brain to allow studies of psychiatric and neurological disorders for which animal models are not available, as altered signal-transduction systems have been implicated in several of these disorders [3–5].

A new method to measure phosphoinositide metabolism in human postmortem brain has recently proven successful [6,7] using techniques developed by Claro and colleagues [8]. This method uses membranes prepared from brain incubated with exogenous labelled phosphoinositides. Several different agonists effectively stimulated receptors coupled to G-proteins which activate membrane-bound phospholipase C to cleave the added phosphoinositides. The utilization of exogenous labelled phosphoinositides overcomes the inability to label endogenous phosphoinositides in human postmortem brain slices using conventional methods [2]. Although with this method intact cells cannot be used and not all agents that are stimulatory in animal brain slices activate the system in membranes, this method provides the first technique that enables measurements of receptor-coupled G-protein-dependent phosphoinositide hydrolysis in postmortem human brain [6,7]. Analysis of G-protein and phospholipase C functions revealed no loss in activity with postmortem delays of 6–21 h in membranes prepared from postmortem human prefrontal cortex, supporting the validity of using postmortem tissue [7]. Several agonists activated this system in the presence of guanosine 5′-*O*-(thiotriphosphate) (GTP[S]), supporting the conclusion that the entire second-messenger system composed of receptors, G-proteins and phospholipase C can retain activity in postmortem human brain membranes. In the present paper we identify further active components of the phosphoinositide system in membranes prepared from postmortem human brain.

MATERIALS AND METHODS

Frozen tissue samples from postmortem human brains were obtained from the University of Alabama at Birmingham Brain Resource Program directed by R.P. All subjects were free of neurological pathology upon postmortem examination. The prefrontal cortex from six subjects was used, three male and three female, 72 ± 8 years of age (mean ± S.E.), with a 12.8 ± 0.5 h postmortem interval.

Membranes were prepared and Ptd[^{3}H]Ins hydrolysis was measured as described previously [9,10]. Final incubation mixtures (0.1 ml) contained 0.1 mg of membrane protein, 10 mM Tris/maleate, pH 6.8, 6 mM $MgCl_2$, 8 mM LiCl, 3 mM EGTA and sufficient $CaCl_2$ to yield a free Ca^{2+} concentration of 0.3 μM, 1 mM deoxycholate, 0.1 mM [^{3}H]PtdIns [(5–10) × 10^4 c.p.m./10 nmol; New England Nuclear, Boston, MA, U.S.A.], and other additions as indicated. In experiments with antibodies, membranes were preincubated for 30 min at room temperature with a polyclonal antibody to the G-proteins $G_{\alpha q/11}$ (1:100, v/v; anti-G-protein QL, New England Nuclear), with antibodies to subtypes of phospholipase C (1:50, v/v; Upstate Biotechnology Inc., Lake Placid, NY, U.S.A.), or with an equal dilution of control rabbit serum (1:100, v/v). Reactions continued for the stated times at 37 °C, and were stopped by addition of 1.2 ml of chloroform/methanol (1:2, v/v). Chloroform

Abbreviations used: ACPD, *trans*-1-aminocyclopentyl-1,3-dicarboxylate; AppNHp, 5′-adenylyl-$\beta\gamma$-imidodiphosphate; GTP[S], guanosine 5′-*O*-(thiotriphosphate); 5HT, 5-hydroxytryptamine.

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(0.5 ml) and 0.25 M HCl (0.5 ml) were added, samples were mixed and placed on ice for 10 min, phases were separated by centrifugation, the aqueous phase was counted for radioactivity, and the pmoles of [^{3}H]PtdIns hydrolysed were calculated based upon the specific activity of [^{3}H]PtdIns. Each assay was carried out in triplicate and analysis of variance was used to determine statistical significance.

RESULTS

Agonist-induced [^{3}H]PtdIns hydrolysis

The relative effects of GTP[S] and four agonists on [^{3}H]PtdIns hydrolysis in six different preparations of human prefrontal cortical membranes are reported in Table 1. As reported previously [7] GTP[S] (3 μM) significantly stimulated [^{3}H]PtdIns hydrolysis and this was increased further with the addition of each agonist. 5-Hydroxytryptamine (5HT) and ATP produced the largest stimulations in the presence of GTP[S] and these agents also caused a small increase in [^{3}H]PtdIns hydrolysis in the absence of GTP[S]. Therefore the responses to ATP and 5HT were examined in further detail.

Responses to ATP and serotonin

Figure 1 shows the time course of the stimulation of [^{3}H]PtdIns hydrolysis by GTP[S] alone and with ATP (Figure 1a) or with 5HT (Figure 1b). All rates of hydrolysis were approx. linear through 60 min of incubation, except for some slowing of activity with 1 mM ATP plus GTP[S] after 30 min. The lower concentrations of ATP (0.1 mM) or of 5HT (0.3 mM) each in the presence of GTP[S] only slightly elevated [^{3}H]PtdIns hydrolysis above that caused by GTP[S] alone, whereas 1 mM ATP or 5HT with GTP[S] induced a robust response.

We reported previously [7] that an antibody to the α-subunit of the G-protein $G_{q/11}$, but not antibodies to other α-subunit subtypes, blocked the stimulation by several different agonists of [^{3}H]PtdIns hydrolysis in human membranes, but ATP was not tested in those experiments. To determine whether [^{3}H]PtdIns hydrolysis induced by ATP in the presence or absence of GTP[S] was mediated by the phospholipase-C-linked G-protein $G_{q/11}$, membranes were incubated with a polyclonal antibody to $G_{q/11}$ or with serum as a control. Figure 2 shows that anti-$G_{q/11}$ virtually eliminated the response to GTP[S] alone, and blocked the GTP[S]-dependent portion of the response to the combination of ATP plus GTP[S]. [^{3}H]PtdIns hydrolysis stimulated by ATP in the absence of GTP[S] was unaffected by the $G_{q/11}$ antibody. Therefore, GTP[S]-dependent [^{3}H]PtdIns hydrolysis stimulated by ATP in human membranes is primarily mediated by $G_{q/11}$, but the hydrolysis induced by ATP in the absence of GTP[S] is mediated by other processes. To test whether [^{3}H]inositol polyphosphates were produced as a consequence of ATP-supported phosphorylation of [^{3}H]PtdIns, they were examined by ion-exchange chromatography but were found not to accumulate (results not shown), consistent with their rapid hydrolysis by brain membranes reported previously [11].

The antibody to $G_{q/11}$ also was tested on 5HT-stimulated [^{3}H]PtdIns hydrolysis. Anti-$G_{q/11}$ reduced the stimulation induced by 0.3 mM 5HT plus GTP[S] to the level obtained in the absence of GTP[S] (Figure 2). With 1 mM 5HT in the presence of GTP[S], the anti-$G_{q/11}$ blocked most, but not all, of the GTP[S]-dependent portion of the stimulation. The response to 5HT obtained in the absence of GTP[S] was unaffected by the anti-$G_{q/11}$. Thus, as with ATP, $G_{q/11}$ mediated GTP[S]-dependent, but not GTP[S]-independent, 5HT-induced hydrolysis of [^{3}H]-PtdIns in human brain membranes.

Table 2 shows the effects of several adenosine-containing

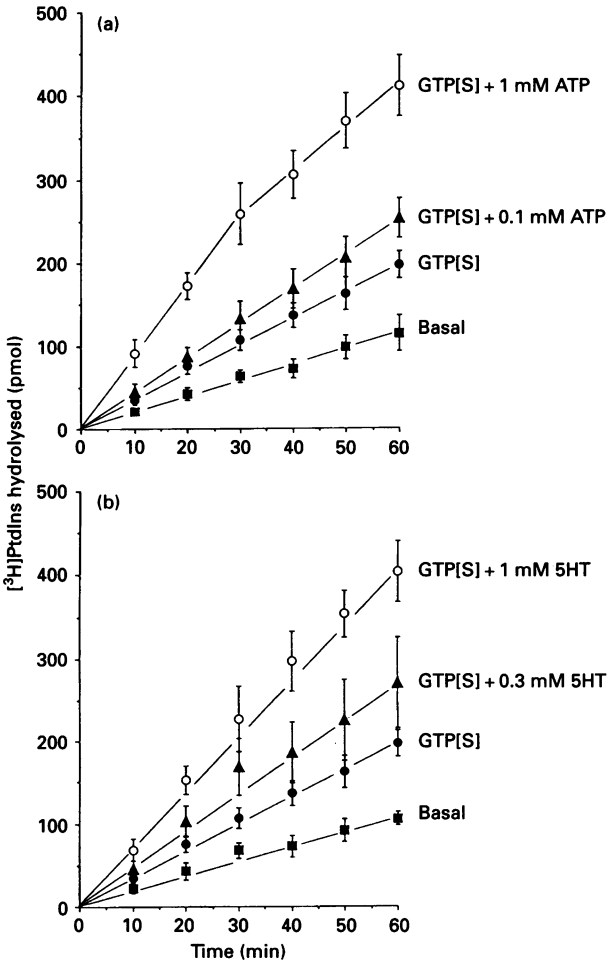

Figure 1 Time course of [^{3}H]PtdIns hydrolysis with (a) ATP or (b) 5HT

Human prefrontal cortical membranes were incubated with [^{3}H]PtdIns at 37 °C for 10–60 min in the absence of added agents (basal), with 3 μM GTP[S], with 0.1 mM or 1 mM ATP plus GTP[S], or with 0.3 mM or 1 mM 5HT plus GTP[S]. Data represent means ± S.E.M. (n = 3–5).

Table 1 Comparison of [^{3}H]PtdIns hydrolysis in human brain membranes stimulated by four agonists

Human prefrontal cortical membranes were incubated with [^{3}H]PtdIns for 20 min at 37 °C in the absence (−) or presence (+) of 3 μM GTP[S] and with 1 mM carbachol, ACPD, 5-hydroxytryptamine (5HT) or ATP. Data represent means ± S.E.M. (n = 6). *P < 0.05 compared with the basal value obtained in the absence of agonist and GTP[S]; **P < 0.05 compared with the value obtained with GTP[S] alone.

	[^{3}H]PtdIns hydrolysis (pmol)	
Agonist	−GTP[S]	+GTP[S]
None	34 ± 4	54 ± 7*
Carbachol	37 ± 4	71 ± 8**
ACPD	46 ± 6	87 ± 13**
5HT	49 ± 5*	109 ± 10**
ATP	71 ± 8*	143 ± 10**

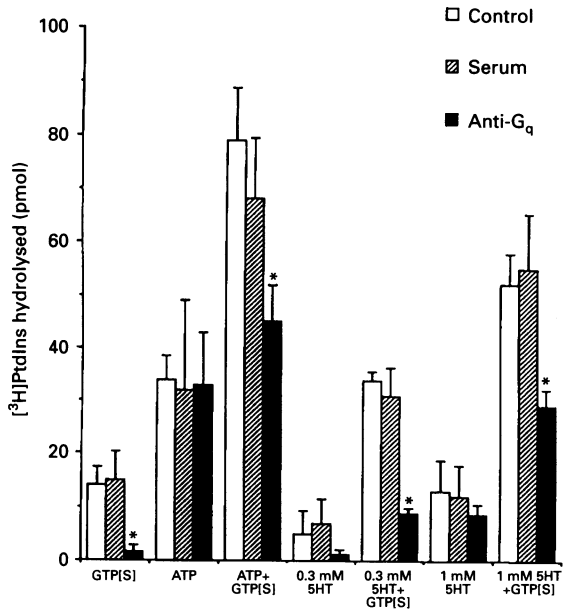

Figure 2 Effect of $G_{q/11}$ antibody on [^{3}H]PtdIns hydrolysis

Human prefrontal cortical membranes were incubated with [^{3}H]PtdIns for 20 min at 37 °C without additions (basal) or with 3 μM GTP[S], 1 mM ATP, GTP[S] plus ATP, 0.3 mM or 1 mM 5-HT, each alone or plus GTP[S], after preincubation with a $G_{q/11}$ antibody, with rabbit serum, or without additions (control). Basal values were subtracted to obtain the GTP[S]- or agonist-induced hydrolysis. Data represent means ± S.E.M. ($n = 3$). *$P < 0.05$ compared with control for each treatment.

Table 2 Specificity of ATP-stimulated [^{3}H]PtdIns hydrolysis

Human prefrontal cortical membranes were incubated with [^{3}H]PtdIns for 40 min at 37 °C in the absence (−) or presence (+) of 3 μM GTP[S] and without (basal) or with 1 mM adenosine, AMP, AppNHp, ADP or ATP. Basal values were subtracted to calculate the stimulation induced by each agent. Data represent means ± S.E.M. ($n = 3$). *$P < 0.05$.

	[^{3}H]PtdIns hydrolysis (pmol)	
Treatment	−GTP[S]	+GTP[S]
Basal	−	31 ± 5
Adenosine	3 ± 2	20 ± 3
AMP	2 ± 2	37 ± 2
AppNHp	6 ± 2	39 ± 6
ADP	36 ± 3*	105 ± 6*
ATP	99 ± 3*	180 ± 2*

compounds on [^{3}H]PtdIns hydrolysis. ATP produced the greatest stimulation of [^{3}H]PtdIns hydrolysis among the agents tested, and ATP had two effects: it was stimulatory both in the absence and presence of GTP[S]. ADP also induced [^{3}H]PtdIns hydrolysis, but 5′-adenylyl-$\beta\gamma$-imidodiphosphate (AppNHp), AMP, and adenosine were without effect.

To identify further the mechanisms mediating [^{3}H]PtdIns hydrolysis induced by ATP, the stimulation induced by ATP was compared with that of 2-methylthio-ATP, a selective P_2-purinergic receptor agonist. In the absence of GTP[S], ATP but not 2-methylthio-ATP stimulated [^{3}H]PtdIns hydrolysis (Table 3). In the presence of 3 μM GTP[S], stimulation of [^{3}H]PtdIns hydrolysis by 2-methylthio-ATP was 73 % of that induced by ATP,

Table 3 P_2-purinergic receptor-stimulated [^{3}H]PtdIns hydrolysis

Human prefrontal cortical membranes were incubated with [^{3}H]PtdIns for 40 min at 37 °C in the absence or presence of 3 μM GTP[S] and with 1 mM ATP or 2-methylthio-ATP (2mATP), each after a 30 min preincubation with or without $G_{q/11}$ antibody. Values obtained without added GTP[S] or agonist (basal; 50 ± 8.1 pmol) were subtracted from those with agonist alone to calculate the GTP[S]-independent stimulation, and values obtained with the agonist alone in the absence of GTP[S] (GTP[S]-independent) were subtracted from those with agonist plus GTP[S] to calculate the GTP-dependent stimulation. Data represent means ± S.E.M. ($n = 4$).

		[^{3}H]PtdIns hydrolysis (pmol)	
Agonist	Anti-$G_{q/11}$	GTP[S]-independent	GTP[S]-dependent
None	−	−	33 ± 10
ATP	−	48 ± 15	127 ± 37
ATP	+	−	21 ± 18
2 mATP	−	2 ± 5	93 ± 29
2 mATP	+	−	34 ± 13

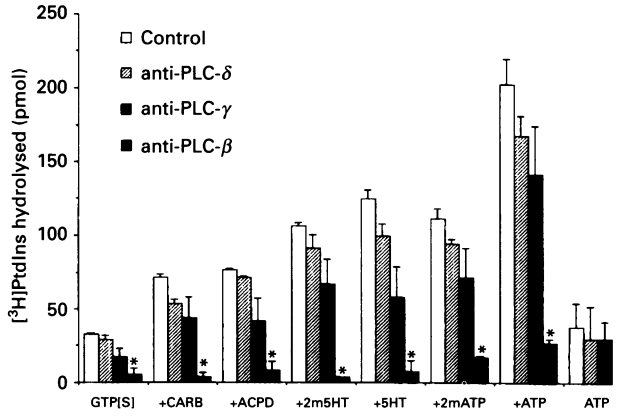

Figure 3 Effects of phospholipase C subtype antibodies on [^{3}H]PtdIns hydrolysis

Human prefrontal cortical membranes were incubated with [^{3}H]PtdIns for 20 min with 3 μM GTP[S] alone or with (+) 1 mM carbachol (CARB), ACPD, 2-methyl-5HT (2m5HT), 5HT, 2-methylthio-ATP (2mATP), ATP, or with ATP alone, after preincubation with antibodies to the indicated subtypes of phospholipase C (PLC) or rabbit serum as a control. Basal values were subtracted to obtain the GTP[S]- or agonist-induced hydrolysis. Data represent means ± S.E.M. ($n = 3$). *$P < 0.01$ compared with control.

and inclusion of an antibody to $G_{q/11}$ blocked the GTP[S]-dependent responses to both ATP and 2-methylthio-ATP. From these results it can be concluded that P_2-purinergic receptors coupled to $G_{q/11}$ are primarily responsible for GTP[S]-dependent ATP-stimulated [^{3}H]PtdIns hydrolysis in postmortem human brain membranes.

Phospholipase C

Three families of phospholipase C have been identified and classified as δ, γ and β [13]. Antibodies specific for each of these subtypes were incubated with human brain membranes before measuring agonist-induced [^{3}H]PtdIns hydrolysis to identify which class of phospholipase C mediated this process. Pre-incubation with the antibody to phospholipase C-β_1 almost completely blocked [^{3}H]PtdIns hydrolysis induced by GTP[S] or by GTP[S] plus carbachol, *trans*-1-aminocyclopentyl-1,3-dicarboxylate (ACPD), 2-methyl-5HT, 5HT, 2-methylthio-ATP, or ATP (Figure 3). The antibody to phospholipase C-δ_1 was without

effect and although the antibody to phospholipase C-γ_1 in some cases appeared to cause some reduced responses, these effects were not statistically significant. Stimulation of [^{3}H]PtdIns hydrolysis by ATP in the absence of GTP[S] was not significantly inhibited by any of the phospholipase C antibodies. These results demonstrate that phospholipase C-β_1 is the primary subtype of phospholipase C that mediates agonist-induced, GTP[S]-dependent [^{3}H]PtdIns hydrolysis in human brain membranes.

DISCUSSION

Phosphoinositide metabolism is recognized as a major signal-transduction system in mammalian brain as a result of many studies using tissues from laboratory animals [1]. Comparable investigations in postmortem human brain have been prevented because postmortem delays precluded utilization of the classical techniques of labelling phosphoinositides in slices with [^{3}H]inositol or [γ-^{32}P]ATP. The use of exogenous labelled phosphoinositides as substrates with brain membranes overcomes that obstacle to some extent [6,7]. Although there are limitations with this method, including the artificial environment of the substrate created by using exogenous lipids, the use of [^{3}H]PtdIns rather than [^{3}H]PtdIns(4,5)P_2 as substrate, and the lower magnitudes of stimulation compared with those obtained in fresh animal brain slices, the capability of measuring the activity of the entire coupled complex of receptor, G-protein and phospholipase C in postmortem human brain membranes affords an opportunity to study this system in human brain tissue and to identify the effects of diseases for which adequate animal models do not exist.

Of the agonists that have been identified as causing stimulation of [^{3}H]PtdIns hydrolysis by postmortem human brain membranes [7], carbachol acts through muscarinic receptors and ACPD stimulates glutamate metabotropic receptors, but the mechanism mediating the response to ATP had not been studied. Two responses to ATP were distinguished: (a) a relatively large GTP[S]-dependent hydrolysis of [^{3}H]PtdIns, and (b) some [^{3}H]PtdIns hydrolysis in the absence of GTP[S]. The GTP[S]-independent component of the response to ATP appears likely not to be mediated by activation of a receptor·G-protein complex because it was not impaired by an antibody to $G_{q/11}$ and was not induced by 2-methylthio-ATP. Two mechanisms are likely to contribute to the GTP[S]-independent hydrolysis of [^{3}H]PtdIns induced by ATP: phosphorylation of [^{3}H]PtdIns and direct activation of phospholipase C. Wallace et al. [14] have reviewed evidence that brain membranes retain PtdIns kinase and PtdIns4*P* kinase and that incubation with ATP supports the phosphorylation of the phosphoinositides. It is likely that labelled PtdIns4*P* and PtdIns(4,5)P_2 are produced from [^{3}H]PtdIns in the presence of ATP and are subsequently hydrolysed at a slow, basal rate, as is [^{3}H]PtdIns, and thereby contribute to the GTP[S]-independent increase in hydrolysis occurring in the presence of ATP [8]. In addition, others have reported that ATP can directly activate phosphoinositide-linked phospholipase C [15,16], and this could also contribute to the GTP[S]-independent stimulation of [^{3}H]PtdIns hydrolysis induced by ATP.

Of greater interest is the finding of a relatively large GTP[S]-dependent stimulation of [^{3}H]PtdIns hydrolysis induced by ATP. This response to ATP appears to be mediated by a P_2-purinergic receptor linked to $G_{q/11}$ because [^{3}H]PtdIns hydrolysis was also stimulated by ADP and by 2-methylthio-ATP, and because an antibody to $G_{q/11}$ inhibited the GTP[S]-dependent [^{3}H]PtdIns hydrolysis stimulated by ATP and by 2-methylthio-ATP. This conclusion is in accordance with previous results from a number of other tissues that demonstrated that the P_2-purinergic receptor mediated the stimulation by ATP of phosphoinositide-linked phospholipase C [1]. The presence in brain membranes of kinases that catalyse phosphorylation of phosphoinositides when ATP is available [14] may explain the relatively larger response obtained with ATP than with other agonists in the presence of GTP[S]. Under this condition, each of the phosphoinositides may be labelled and contribute to phospholipase-C mediated hydrolysis, although the presence of inositol phosphatases subsequently leads to accumulation of primarily inositol monophosphates (R. S. Jope, L. Song and R. Powers, unpublished work) [11]. However, we have not detected increased products induced by other agonists when ATP was included in the incubation medium (R. S. Jope, L. Song and R. Powers, unpublished work). Regardless of the mechanism, stimulation of P_2-purinergic receptors produces a relatively strong GTP[S]-dependent response when postmortem human brain membranes are incubated with [^{3}H]PtdIns.

In animal brains, phospholipase C-β appears to be the major isozyme of phospholipase C responsible for the hydrolysis of phosphoinositides following activation of receptors coupled to heterotrimeric G-proteins [13]. For example, antibodies specific for phospholipase C-β blocked phosphoinositide hydrolysis induced by GTP[S] and carbachol in rabbit cortical membranes [18]. Results reported here demonstrate that, in human cortical membranes, phospholipase C-β is also the major isozyme of phospholipase C responsible for [^{3}H]PtdIns hydrolysis induced by GTP[S] and each of the several agonists that were examined. This finding is consistent with the partial membrane-bound localization of phospholipase C-β [13,19], but does not preclude the interaction of cytosolic phospholipase C subtypes with phosphoinositide hydrolysis in intact cells, as those enzymes would have been excluded from the membrane preparation used in this assay.

Taken together with previous reports [6,7], the results of this study further our knowledge of the utility of assaying [^{3}H]PtdIns hydrolysis by membranes prepared from postmortem human brain. Although PtdIns(4,5)P_2 rather than PtdIns is thought to be the primary endogenous substrate, and direct comparisons of the hydrolysis of [^{3}H]PtdIns and [^{3}H]PtdIns(4,5)P_2 should provide useful information for evaluating this system in human tissue, PtdIns itself has been reported to be an endogenous substrate of phospholipase C (e.g. [20]), and measurement of [^{3}H]PtdIns hydrolysis appears to meet the appropriate criteria as a relevant assay of the activation of the phosphoinositide second-messenger system in human brain membranes. Phospholipase C-β and $G_{q/11}$ were identified as the primary subtypes of phospholipase C and heterotrimeric G-protein respectively, that couple agonist-induced receptor activation to phosphoinositide hydrolysis, findings that are consistent with results using other tissue sources. Therefore the use of these methods should provide an avenue for studying the modulation of phosphoinositide hydrolysis in human brain and alterations associated with psychiatric and neurological disorders.

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